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## Targeting microRNAs in Pancreatic Cancer: Microplayers in The Big Game

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### Abstract

The prognosis of patients with pancreatic cancer is extremely poor, and current systemic therapies result in only marginal survival rates for patients. The era of targeted therapies has offered a new avenue to search for more effective therapeutic strategies. Recently, microRNAs (miRNAs) that are small non-coding RNAs (18–24 nucleotides) have been associated with a number of diseases including cancer. Disruption of miRNAs may have important implications in cancer etiology, diagnosis and treatment. So far, focus has been on the mechanisms that are involved in translational silencing of their targets to fine tune gene expression. This review summarizes *the approach* for rational validation of selected candidates that might be involved in pancreatic tumorigenesis, cancer progression and disease management. Herein, we also focus on the major issues hindering the identification of miRNAs, their linked pathways, and recent advances in understanding their role as diagnostic/prognostic biomarkers and therapeutic tools in dealing with this disease. miRNAs are expected to be robust clinical analytes, valuable for clinical research and biomarker discovery.

### Keywords

Pancreatic Cancer; MicroRNA; Diagnostics; Delivery; Therapeutics

### Pancreatic cancer and miRNA

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States (1). The recent sequencing of the pancreatic cancer genome has underscored the considerable heterogeneity of somatic DNA alterations and dysregulation of genetic pathways between individual tumors and hence challenges for molecularly targeted therapies. The progression model of PDAC is distinguished by three PDAC precursor lesions: pancreatic intraepithelial neoplasia (PanIN I, II, III), a less common precursor lesion mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN) (1). PanIN, which is found in the smaller-caliber pancreatic ducts, is the most common and

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### Potential conflicts of interest

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extensively studied lesion due to the suggested biologic relationship and common genetic alterations documented for initial increased incidence of PanINs in patients with PDAC. Key shared genetic alterations associated with PDAC progression include earliest genetic events such as mutation of K-RAS and overexpression of HER-2/neu. At later stages, inactivation of the p16 tumor suppressor gene occurs, followed by the loss of p53, SMAD4, and BRCA2 signaling pathways and the genomic-transcriptomic alterations that facilitate cell-cycle deregulation, cell survival, invasion, and metastases (1). Therefore, detecting molecular alterations at pre-cancerous conditions together with targeting *PDAC* at an early stage represent an assault on the Achilles heel of cancer. Recently, miRNA therapy has emerged as a promising therapeutic strategy for various diseases. miRNAs are small non-coding RNAs consisting of 18–24 nucleotides that regulate their target mRNA stability and translation by binding imperfectly with their 3' UTR region. Apart from the role of miRNAs in cancer progression and invasion, their differential expression has been associated with patient survival and regulation of the disease response (resistance or sensitization) towards chemotherapeutic drugs.

The clinical potential of miRNAs is based on the fact that a single miRNA can regulate multiple oncogenic pathways commonly deregulated in cancer. A uniform pattern of miRNA dysregulation in PDAC is still lacking. This is due to the highly heterogeneous nature of pancreatic tissue which contains not only pancreatic ducts and acinar cells from which ductal type tumors may arise, but also the predominance of dense desmoplastic non neoplastic stromal, and infiltrating inflammatory cells. miRNAs can regulate several molecules, including mucins that harbor the complex tumor-stromal microenvironment and contribute to tumor progression and chemo-resistance. Thus, the rapid and coordinated manipulation of protein levels across multiple pathways endows these regulatory RNAs with the ability to instantly switch between cellular programs. Also, the identification of miRNAs during transition from PanIN I, PanIN II and PanIN III to adenocarcinoma and the specific roles they exert in such a process is warranted to properly design strategies to prevent and/or attenuate the malignant phenotype. Thus far, very few studies have investigated miRNA changes associated with PDAC progression. This review summarizes the strategies to tackle PanCa at genetic level through the miRNAs.

## Rationale for developing miRNA based therapy

Considerable research shows that restoring the expression of Tumor suppressor-miRNAs seeks to reinstate those cellular programs that are active in normal cells and interfere with oncogenic programs necessary for a malignant phenotype. However, antagonizing the function of over-expressed oncogenic miRNAs inhibits multiple oncogenic pathways. It has been successfully shown that intravenous injection of antagomirs, 2'-O-methyl-antisense oligonucleotides conjugated with a cholesterol moiety at the 3'-end, leads to long-lasting inhibition of specific miRNAs in mice (2). In contrast, restoration of expression of down-modulated tumor-suppressive miRNAs is usually achieved using adeno- and lenti-viral vectors, non-viral lipid-based strategies that have recently been developed for systemic miRNA delivery and applied successfully to lung and prostate cancer xenograft models (3). Miravirsin (LNA oligomer targeting miR-122), after showing positive effects against HCV infection in Chimpanzees (4), showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance in patients with chronic HCV genotype 1 infection (5). Standard computational analysis is utilized in a combined Genome-Wide Association Study (GWAS) miRNA profiling for the identification of response predictors in metastatic breast cancer patients (NCT01598285, ClinicalTrial.gov). The success of preclinical trial of MRX34 (proprietary tumor suppressor miRNA, miR-34) in an orthotopic mouse model of hepatocellular carcinoma through the SMARTICLES oligonucleotide delivery technology has prompted the start of its Phase 1 clinical trials (Mirna Therapeutics,

Inc) recently, in may 2013 <http://www.clinicaltrials.gov/ct2/show/NCT01829971?term=MRX34&rank=1>. miR-34-based therapy and the MRX34 is the first miRNA mimic to advance into a human clinical trial. Similar approaches can be used to target the highly fibrotic pancreatic tumors. Overall, these strategies, besides being powerful approaches for functional validation of miRNAs relevant for a specific disease, constitute a prerequisite for the development of potential miRNA-based therapies. Herein, we discuss several important miRNAs which have consistently been shown to be involved in PDAC progression by many research groups. Also, we discuss strategies of miRNA modulator delivery in cancer models, a subject that remains the key challenge to the establishment of miRNA therapeutics.

## miRNAs in pancreatic cancers. Where are we now?

The functional activity of only a handful of miRNAs has been experimentally modeled in the context of PanCa. Some of these miRNAs have been characterized as potent oncogenes (oncomiRs) or tumor suppressors (tsmiRs), based on the consequences of their expression on the phenotype in experimental models.

### Oncogenic microRNAs

miRNA profiling and functional studies reveal several deregulated miRNAs in PDAC progression but very few studies have demonstrated their role in the key shared genetic alterations associated with their precursor lesions. The most specifically overexpressed miRNA in PanIN III lesions (carcinoma *in situ*) identified is miRNA-196b (6). Aberrant expression of several miRNAs has been identified early in PanIN lesions as well as throughout the multistep progression of PDAC. They include miR-21, miR-155, and miR-221 (7) which have been linked to fibrogenesis through transforming growth factor (TGF- $\beta$ ) that promotes their expression. They play an important role in functioning as proto-oncogenes and have been associated with decreased survival in PDAC patients (8). miR-155 targets a key tumor suppressor, TP53INP1 (9), which is expressed in normal tissues in early stages of PanCa development. miR-21 decreases the expression of SMAD7, a negative regulator of TGF- $\beta$  signaling (10). On the other hand, several studies have reported significant overexpression of miR-21 in PDAC associated with chemo-resistance (11). Thus, inhibition of miR-21 in PDAC would be useful to inhibit the *desmoplastic reactions potentially via repression of TGF- $\beta$  signaling* in PDAC (12) to overcome drug resistance and inhibit tumor surveillance.

Immunosuppressive tumor microenvironments can restrain antitumor immunity, particularly in PDAC. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. miR-224 and miR-486 are upregulated and target CD40, thus downregulating its protein expression at cell surfaces in highly invasive and metastatic PDAC (13). The oncogenic role of selected miRNAs has mainly been associated with their ability to enhance the proliferative potential of cancer cells through the suppression of cell-cycle-related protein pRb (14), as well as members of the E2F family of transcription factors. For instance, miR-132 and miR-212 are upregulated in PDAC tissues and these miRNAs promote tumorigenesis by reducing pRB, thus increasing expression of E2F gene products (14).

A critical tumor suppressor, DPC4/Smad4, is involved in the progression of PDAC and is targeted by miR-27a (15). Attenuation of miR-27a in PDAC cells, reduced growth, colony formation, migration, and also down regulated sprout2 (*spry2*), a negative feedback regulator of multiple receptor tyrosine kinases exerting an oncogenic effect. MicroRNA-10 a/b are overexpressed in PDAC, promote its invasiveness, and correlate with a poor prognosis (16–18). HOX genes have been identified as their potential targets in many cancers, including PDAC (19). An association between aberrant miRNA expression and

malignancy in PDAC was recently shown with an oncogenic positive feedback loop. miR-301a inhibits pro-apoptotic Bim expression (20) and suppresses NF- $\kappa$ B repressing factor (NKRF) in PDAC, leading to NF- $\kappa$ B activation, which in turn activates the transcription of miR-301a. Inhibition of miR-301 or overexpression of NKRF reverses the process and slows down tumor growth, suggesting that miR-301a contributes to oncogenesis by activating NF- $\kappa$ B (21). In addition, increased expression of miR-155, miR-21, miR-203, miR-210, and miR-222 has reportedly been associated with poor survival in cancer patients (8). Moreover, high expression of miR-196a-2 is associated with patients' poor outcome (22), which suggests it may be a prognostic marker for PDAC. Additionally, applying silencing strategies to these miRNAs may likely alter the outcome of conventional therapeutics and overall survival of PanCa patients.

### Tumor suppressor microRNAs

Recently, the miR-200 a/b/c family has been observed to suppress stemness of cancer cells by suppressing stem cell factors such as Sox2 and inhibiting SIP1 and ZEB1 (negative regulator of E-cadherin), thereby inhibiting EMT and invasion in PanCa cells (23). But the miR-200 family (overexpressed in PanINs/miR-200c only in low grade PanINs), including Let-7, miR-194 and miR429, are found to be significantly overexpressed with epigenetic alterations in highly invasive and metastatic PDAC targeting the tumor and metastasis suppressor, EP300 mRNA and protein (24). Thus, the epigenetic modification responsible for abnormal expression of miRNAs in pancreatic tumors may be a crucial mechanism contributing to cancer invasion and metastasis. Differential expression of certain miRNAs detected in PanINs correlates with their expression in invasive PDAC.

A well-documented tumor suppressor in PDAC is miR-375, which targets 14-3-3 zeta (15, 22) and induces caspase dependent apoptosis. Its overexpression downregulates the PDK1/Akt survival pathway and leads to downregulation of potential biomarkers such as IGFBP5 and CAV-1 (25). Very recently, diverse tumor-suppressive functions have been associated with miR-15a which modulates the survival pathways by targeting WNT3A (Wnt/  $\beta$ -catenin pathway) and FGF7 (26). Ectopic expression of miR-96 increases cell apoptosis reduces migration, proliferation, and invasion of PDAC cell lines by inhibition of the KRAS/Akt (27). Additionally, the downregulation of miR-34a is seen along with the loss of p53 function (28) implicating its role in PDAC.

### Therapeutic implications of miRNAs, a way towards the clinic

The therapeutic application of miRNAs involves two strategies. One strategy is directed against a gain-of-function and aims to inhibit oncogenic miRNAs by using miRNA antagonists. The second strategy against a loss-of-function, miRNA replacement, involves the re-introduction of a tumor suppressor miRNA to restore a loss of function.

### miRNA gain-of-function

It is our understanding that miR-21, miR-155, miR-196a, miR-210 and miR-221 (Table 1) are overexpressed in PDAC and currently represent the most promising candidates as oncogenic miRNAs.

### Antisense oligonucleotides (ASOs)

It has been shown that ASOs can inhibit up regulated oncogenic miRNAs in tumors in a specific, efficient and long-lasting manner (29). ASOs for miR-21 and miR-221 increase the levels of their targets (PTEN, RECK and CDKN1B), reduce proliferation and increase apoptosis of PanCa cells (30, 31). ASOs also sensitize PDAC cells to Gemcitabine and generate synergistic anti-tumor effects. Preclinical studies have shown potent activity of

AEG35156 (targets X-linked inhibitor of apoptosis mRNA) in combination with gemcitabine in PDAC (32). These results imply that targeting miRNAs with ASOs could be a potential new therapeutic strategy for PDAC.

### AntagomiRs (LNA and AMO)

The miRNA antagonists carry chemical modification that degrades and traps the endogenous miRNA in a configuration that is unable to be processed by RISC. A locked nucleic acid (LNA) is an ASO modified by LNA technology (bicyclic high-affinity RNA mimicking N-type (C3'-endo) conformation by the introduction of a 2'-O,4'-C methylene bridge). An anti-miRNA oligonucleotide (AMO) is a single-stranded ASO, chemically modified (usually with a 2'-O-methyl group) 17 to 22 nucleotide long nucleic acid molecule. A study showed that an antagomir against miR-21 sensitized cultured cells to 5-FU treatment, suggesting this miRNA therapy is a promising treatment for tumors that are resistant to 5-FU (11). Also, MTg-AMO (multiple-target AMO) is an innovative approach in which multiple antisense units are engineered into a single unit for simultaneous silencing of multiple-target miRNAs (33). Studies suggest that the MTg-AMO is an improved approach for miRNA target finding and miRNA function validation (34). The combined targeted effect against the oncomirs will validate the physiological relevance of their ability to inhibit invasion or metastasis and the development of miRNA-based therapeutic modality for PDAC.

### Agents targeting epigenetic alterations

Studies have shown that subsets of miRNAs are also modulated by aberrations in epigenetic regulation. Studies show garcinol [a potent inhibitor of histone acetyltransferases (HATs)] synergizes with gemcitabine to modulate specific miRNA biomarkers (miR-21, miR-196a, miR-495, miR-605, miR-638, and miR-453) that sensitize PDAC cells to gemcitabine treatment, thus attenuating the drug-resistance phenotype (35). Another study shows that trichostatin A (TSA), a histone deacetylase inhibitor induces downregulation of oncogenic miR-21 and upregulation of tumor suppressor miR-200c in PanCa cells resulting in cell cycle arrest and increased apoptosis (36). Therefore, epigenetic regulation of miRNAs with a histone deacetylase inhibitor may be an effective therapeutic option for PanCa.

### miRNA loss of function

In our opinion, the miRNAs, Let-7, miR-34a, miR-96, miR-375, miR-200 family (Table 1) currently represent the most promising candidates as tumor-suppressive miRNAs.

### miRNA replacement therapy

Efficient reconstitution of miRs through *in vivo* delivery of miRNA precursors is a crucial factor for the development of successful miRNA-based treatment modalities, a practical application of which is still pending. Restoration of lost let-7 expression in gemcitabine-resistant PDAC cells inhibits proliferation, restores epithelial phenotype and renders the tumor cell sensitive to gemcitabine. Also, ectopic expression of members of the let-7 family of miRNAs in cancer cells increased their *in vitro* radiation response (37). Based on this evidence, rational design of combined therapeutic approaches comprising anticancer agents and specific miRNA modulators can be envisaged to improve therapeutic response. Functional studies carried out in PDAC models attribute an oncosuppressive function to miR-34 because it is a key regulator of cancer cell growth and invasion. Restoration of miR-34 can have significant promise as a novel molecular therapy as it reduces tumor initiating cancer stem cells mediated by inhibition of its downstream targets BCL2 and NOTCH (38) and sensitizes to chemo/radiation therapies (87%). Therefore, miR-34 is prospectively potential target to be restored in tumors for modulating many oncogenic signaling pathways. The most commonly used strategies against loss-of-function to probe



miRNA functions in vivo are synthetic miRNA mimics (miRNA overexpression), vector-mediated miRNA over-expression and small molecule therapy.

### Small-molecule drugs targeting specific miRNAs (SMIRs)

From the perspective of their secondary structures, miRNAs appear to be “druggable” targets. The formation of stem loops found in pre-miRNAs and the bulges in miRNAs facilitate small molecule targeting, thereby, making the development of small-molecule drugs (SMIRs) a promising prospect. SMIRs interact and bind to the grooves and pockets on the surfaces of miRNAs directly, thereby interfering with the biological functions of targeted miRNAs and inhibiting the processing of miRNAs (39). A number of agents including isoflavone and 3,3-diindolylmethane have been shown to alter expression of miR-200b, miR-200c, let-7b, let-7c, let-7d, and let-7e in gemcitabine-resistant cancer cells (40). A naturally occurring flavonoid, curcumin (diferuloylmethane) from the rhizome of *Curcuma longa*, has been shown to have tremendous effects on the expression profiles of miRNAs in PanCa models. It causes overexpression of miR-22 and downregulation of miR-199a (41), regulating Sp1 transcription factor (SP1) and estrogen receptor 1 (ESR1) which are involved in cell growth, metastasis and apoptosis.

### Nanovector therapy

Non-viral lipid-based complexes, liposomes and nanoparticles have been promising avenues used for systemic miRNA delivery. Studies show that systemic nanovector therapy modulates direct and indirect miRNA targets in a PanCa xenograft mouse model (42). In our opinion, this is an effective delivery system displaying carrier-defined specificity to deliver miRNA or miRNA-modulating agents to relevant tissues or organs. The SMARTICLES oligonucleotide delivery technology is currently being used to deliver MRX01 (proprietary tumor suppressor miRNA, miR-34) in a hepatocellular carcinoma orthotopic mouse model. Preclinical trials are projected to begin this year (Mirna Therapeutics, Inc.).

### Correlation of miRNAs in clinico-pathological parameters

miRNAs have potential as diagnostic and prognostic biomarkers and as therapeutic targets in cancer. Poor survival of PanCa, especially PDAC, patients is attributed to diagnosis at an advanced stage. Therefore, the development of minimally invasive early detection biomarkers for effective clinical management is urgently needed. A company named Asuragen, has developed a molecular diagnostic test to distinguish between benign and PDAC that can be performed on fine needle aspirate (FNA) biopsies of lesions. This is used in conjunction with indeterminate and benign FNA cytopathology and is based on the expression levels of the seven miRNAs and their scores.

The detection of biomarkers in bodily fluids (blood, sera, plasma and feces) offers the possibility of detecting disease at an early stage (43, 44). However, the diagnostic and prognostic value of circulating miRNAs in PDAC has not yet been abundantly proven. The discovery of serum miRNAs as potential biomarkers overcomes the problem of collecting tissue samples by an invasive process. In our opinion, the combination of multiple serum miRNAs can serve as a more comprehensive indicator for tumor detection than the conventional single protein-based or single carbohydrate molecule-based biomarkers, such as CA19-9 and CEA. A comparison of the miRNA expression patterns in serum and tissue/cells may provide additional evidence supporting the use of circulating miRNAs as reliable diagnostic biomarkers (43). However, past studies have not provided direct evidence that serum miRNAs are actively secreted or passively leaked from tumor cells. Therefore, more research in this area will provide a proof-of-principle approach for the use of miRNAs as a diagnostic tool for early PanCa detection.

## Future perspectives

The contribution of miRNAs to the biology of PDAC is still at an early stage even though evidence of deregulated expression suggests miRNAs possess crucial roles in disease development/progression. Collectively, given the myriad of diagnostic, prognostic and therapeutic opportunities arising from miRNA modulation, the translation of miRNA-targeted therapeutics into the clinic will probably witness rapid progress. However, it remains unclear whether the identification of miRNA associated with the transition of an early PanIN and other premalignant lesions into PDAC prevents and/or modulates the malignant phenotype. Focus is required on the critical shifts of these miRNAs in the behavior of these lesions and how they relate to underlying molecular alterations, checkpoint responses, genomic complexity, and the activation of key signaling cascades. Pathobiological and clinical information, along with the development of uniform standards for miRNA measurement to facilitate comparison across studies, could potentially explain the whole story. Moreover, the role of miRNAs in various genetic networks and regulatory pathways, including the mesenchyme and stroma, needs to be analyzed in larger cohort neoplastic and normal tissues. This will be important for the identification and validation of specific miRNAs as diagnostic/prognostic markers. In addition, studies regarding the mechanisms of widespread miRNA dysregulation are clearly needed to understand the origin of the highly metastatic properties of PDAC and provide insight for the improvement of the prognosis of this fatal disease. From a therapeutic standpoint, more details regarding the role of miRNAs in maintenance of normal cellular homeostasis as well as development of transformed phenotypes would stimulate rational therapeutic benefits. For this purpose, the development of proper *in vivo* experimental models is important to recapitulate *in vivo* miRNA functions. In addition, getting a small RNA to interfere with the specific mRNA in the target tissue at a safe and therapeutic level by systemic administration remains a great challenge. Hence, adequate assessment of the functional effects after miRNA inhibition and the long-term miRNA antagonism in *in vivo* are of key importance for anti-miR-based functions. Owing to the severity and limited treatment paradigms in cancer the higher tolerance provided by regulatory authorities for novel anticancer agents would overcome the time frame barrier for miRNA therapeutics to reach clinic.

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Table 1

Selected miRNA candidates that might be clinically relevant in the management of PanCa

miRNA	Status	Role	Targets	Significance	Ref.
miR-21		O	EGFR, HER2/neu, PDCD4, Bcl2, PTEN, TIMP2, TIMP3	Postresection short DFS/worse OS Gem chemoresistance Biomarker (Dic: plasma, serum, feces)	(11, 27, 28, 42)
miR-155		O	TP53INP1	Postresection poor OS and prognosis	(8, 9)
miR-196a-2, miR-196b		O	HOXB8, ANXA1, HMGA2	Postresection Biomarker (Dic: plasma)	(19, 43-45)
miR-222/221		O	CDKN1B (p27), PUMA, PTEN, Bim	Postresection poor OS and prognosis Gem chemo-resistance Biomarker (Dic: plasma)	(8, 19, 28, 42)
miR-210		O	HOXA1, FGFR1, HOXA9, COX10, E2F3, RAD52, ACVR1B, MINT	Postresection poor OS Biomarker (Dic: plasma, feces)	(8, 19, 42)
miR 10a/b		O	HOXB8 HOXA1	poor OS; Gem chemo-resistance	(15, 16)
miR-375		O	PDK1, 14-3-3zeta	Biomarker (Dic: plasma, feces)	(19, 22, 46)
miR-301a		O	Bim, NKRF	Proliferation and metastasis	(17, 18)
miR-200a/b/c		TS	EP300	Postresection high expression improved OS Gem chemoresensitivity	(19, 21, 37, 47)
miR-34a		TS	Bcl-2	Improved OS after treatment, marker for Gem chemoresensitivity	(35)
Let-7		TS	E2F2, c-Myc, KRAS, MAPK	Enhances chemoresensitivity	(37)
miR-34b		TS	Smad3	Reduces invasion/metastasis	(48)
miR-96		TS	KRAS, AKT	Inhibits tumor growth and invasion	(24)

DFS: Disease free survival; OS: Overall survival; Gem: Gemcitabine; Dic: Detection